Temperature-induced increase in cellular chelating potential associated with reduced thermotolerance

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Abstract

Hot springs panic grass, *Dichanthelium lanuginosum* var *sericeum* (Schmoll), successfully colonizes geo-thermally heated soils that are subject to chronic temperatures ranging from 40 to 50 °C, occurs at altitudes in excess of 2500 m, and tolerates low soil moisture content for extended periods of time. We utilized superoxide dismutase (SOD; EC 1.15.1.1) as an indicator of oxidative stress response to compare the impacts of temperature on wheat (*Triticum aestivum* L.), a cool-season grass, with the temperature responses of three isolates of *D. lanuginosum*. One of the *D. lanuginosum* isolates was collected from a non-thermal site and had reduced capacity to adapt to growth at elevated temperature. Wheat SOD activity in crude leaf extracts was significantly reduced by incubation at 45 °C. In contrast, SOD activity in crude leaf extracts from a thermophilic *D. lanuginosum* isolate increased after incubation at 45 °C. Significant increases in cellular chelating capacity occurred in wheat and the *D. lanuginosum* isolated from a non-thermal environment after plants were exposed to elevated temperatures. Ultra-filtration of leaf extracts through 10 kDa molecular weight cutoff membranes removed much of the chelating activity and restored apparent SOD activity. The *D. lanuginosum* isolates with the greatest thermotolerance retained SOD activity when exposed to elevated temperatures and did not show statistically significant increases in cellular chelation potential. Wheat SOD activity increased in response to heat shock. The chelation activity was associated with low molecular weight components (< 10 kDa) that did not bind C_{18} media. Ultra-filtration removal of these low molecular weight chelating components increased SOD activity and replacement of low molecular weight chelating components with an equivalent amount of EDTA suppressed SOD activity.
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1. Introduction

Plant adaptive responses to soil and air temperatures are critical to survival, plant productivity, and the capacity to compete within the environment. These responses occur at multiple levels of plant organization (Feder and Hofmann, 1999) and include heritable changes in stomatal physiology (Radin et al., 1994), increased photorespiration (Weis and Berry, 1988), altered fatty acid content of chloroplast membranes (Murakami et al., 2000), enhanced resistance to loss of intrinsic protein structure from photo-oxidative damage, and extrinsic stabilizing factors including molecular chaperones, (Feder and Hofmann, 1999; Joshi et al., 1997; Ristic et al., 1998). Despite multiple adaptive responses, most plants suffer reduced productivity when exposed to prolonged temperatures in excess of 35 °C. Certain plants do, however, successfully colonize environments that are characterized by chronic exposure to soil and air temperatures in excess of 40 °C. The mechanisms that permit survival in these environments are poorly understood because relatively little research has addressed adaptive responses of plants from extreme environments.

One chronically warm environment that limits plant colonization is the geo-thermally heated soil adjacent to geysers and hot springs such as those found in Yellowstone National Park in the US. Stout et al. (1997) found that a C_3 grass, *D. lanuginosum* var *sericeum* (Schmoll) was the predominant species in soils adjacent to Yellowstone thermal areas, occurring where rhizosphere temperatures

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ranged from 40 to 57 °C. They suggested that this heat tolerance was a major ecological factor impacting the capacity of the plant to colonize these soils.

The heat tolerance of *D. lanuginosum* var *sericeum* is of special interest because this plant may serve as a model for the eco-physiology of plant competition in extreme or degraded environments. One factor that may contribute to heat tolerance in this species is enhanced resistance to photo-oxidative damage. Heat-associated photo-inhibition occurs in plants when light energy exceeds the electron transport capacity of the plant (Tsang et al., 1991). When this is accompanied by accumulation of increased concentrations of reactive oxygen species (ROS), photo-oxidation occurs. One ROS generated under these conditions is superoxide ($O_2^{\bullet-}$) which can interact with hydrogen peroxide, in the presence of metal ions, in a Haber-Weiss reaction to form highly reactive hydroxyl radicals ($OH^{\bullet}$) as follows:

$$H_2O_2 + O_2^{\bullet-} \overset{Fe^{2+},Fe^{3+}}{\longrightarrow} OH^- + O_2 + OH^{\bullet}.$$
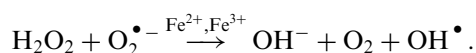
Hydroxyl radicals react with DNA, proteins, lipids, and other cellular constituents to cause oxidative damage associated with certain abiotic stresses (Halliwell, 1987). Plant anti-oxidative defense mechanisms have evolved to convert ROS to less toxic products. Superoxide dismutase (SOD; EC 1.15.1.1) metalloenzymes react with superoxide radicals to produce hydrogen peroxide that can then be removed by catalase (Fridovich, 1995). The three forms of SOD that have been described differ by their metal co-factor and include Cu/Zn-SOD, Mn-SOD and Fe-SOD (Bowler et al., 1992). Over-expression of SOD can enhance plant tolerance to abiotic stresses (McKersie et al., 1993; Perl et al., 1993; Sen Gupta et al., 1993; Van Camp et al., 1994). Accumulation of ROS in wheat chloroplasts can inactivate and degrade Cu/Zn-SOD and presumably reduce abiotic stress tolerance (Casano et al., 1997). The accumulation of relatively low molecular weight compounds that reduce apparent SOD activity in certain heat-treated plants has been demonstrated (Banowetz et al., 2004).

SOD enzyme activity provides an indication of the relative capacity of plants to avoid oxidative damage during abiotic stress. This study utilized SOD activity to compare the relative anti-oxidative capacity of wheat, a cool season grass, with that of three isolates of *D. lanuginosum* after crude leaf extracts and whole plants were exposed to temperatures up to 58 °C. We quantified SOD enzyme activity and demonstrated temperature-associated changes in cellular chelation capacity that apparently impact SOD activity in these two grasses.

2. Materials and methods

2.1. Growth of plant materials

D. lanuginosum and wheat *(Triticum aestivum* L. cv. Stephens) were grown in 10×10 cm plastic pots containing sandy loam with weekly applications of 20:20:20 N:P:K fertilizer. Plants were maintained in a greenhouse with 12 h supplemental light ($300 \,\mu mol \, m^{-2} s^{-1}$). Heat shock and control treatments were conducted at selected temperatures in growth chambers with 12 h photoperiods that provided illumination of $350 \,\mu mol \, m^{-2} s^{-1}$. The three genotypes of *D. lanuginosum* used in these studies included KD8 (collected from a xeric soil at Norris Basin of Yellowstone National Park (YNP)), KD9 (collected from a moist soil near Rabbit Creek in YNP), and KD10 (collected from a non-thermal temperate site at the US Forest Service Susan Creek Campground adjacent to the North Umpqua River in Oregon). Prior to their use as experimental materials, each genotype was grown in the greenhouse for 3–5 years to remove short-term acclimation effects.

2.2. Protein extraction for SOD activity assays

Tissues from fully expanded penultimate leaves (40–80 mg) were excised, weighed, cut to 0.25–0.5 cm strips, placed into 1.0 mL of cooled maceration buffer (50 mM PIPES buffer (pH = 7.5) amended with PVPP ($200 \, mg \, mL^{-1}$)) in a lysing matrix E maceration vial (Bio 101, Carlsbad, CA) and maintained on crushed ice. Tissues were macerated using a FastPrep® FP120 instrument (Bio 101, Carlsbad, CA) at a speed setting of 5.5 for 45 s. The leaf homogenates were centrifuged for 10 min at 12,000g, and supernatants were transferred to microfuge tubes and centrifuged at 14,000g for an additional 30 min. Decanted supernatants were maintained for up to 6 h at 6 °C or used immediately for quantification of total protein and SOD activity. Total protein was measured according to manufacturer's protocol #1 of the NI^{TM} Protein Assay Kit (Geno Technology, Inc., St. Louis, MO).

2.3. SOD assay

SOD assays were conducted as described (Banowetz et al., 2004). Standard curves were prepared utilizing bovine liver SOD (Sigma-Aldrich, St. Louis, MO) in which one unit of SOD corresponded to the amount of enzyme that inhibited the reduction of cytochrome *c* by 50% in a coupled system with xanthine oxidase at pH 7.8 and 25 °C. All SOD determinations were conducted in triplicate, and extrapolated SOD values were expressed as units per mg of total protein per leaf fresh wt (g).

2.4. Ultra-filtration of leaf extracts

The 10,000 molecular weight cutoff (MWCO) retentates were prepared from 250 μL aliquots of leaf extracts using Macrosep® (Pall Filtron Corp.) centrifugal concentrators at 10,000g for 10 min followed by a 200 μL wash with 50 mM PIPES buffer and a second 10,000g centrifugation for 10 min. The filter retentate was back-spun into a clean microcentrifuge tube and brought to a final volume of 250 μL with PIPES buffer. The 10,000 MWCO filtrate, in

contrast, consisted of material which passed the 10,000 MWCO ultra-filtration membranes.

2.5. Quantification of cellular Cu chelating capacity

Chelating capacity of crude leaf extracts was estimated using a Cu-chrome azurol (Cu-CAS) reagent prepared as described (Shenker et al., 1995). Leaf extracts were prepared as described for SOD quantification, and 250 μL aliquots were fractionated by ultra-filtration through 10,000 MWCO Macrosep® (Pall Filtron Corp.) centrifugal concentrators. Five microliters aliquots of the filtrate were diluted with 45 μL dH₂O in each well of a 96 well plate with three replicates per treatment. A standard curve was developed by addition of 50 μL of known concentrations (0–125 μM) of EDTA pH = 8.0 to triplicate wells. Fifty microliters of Cu-CAS indicator dye containing 200 μmol CuCl$_2$ (pH = 5.7) were added to each well, the plates were incubated for 1 h at room temperature, and the absorbance at 595 nm was determined. Standard curves were developed utilizing EDTA as a standard, and chelating activity in samples calculated from the regression equation as EDTA equivalents (μmol mg protein^{-1} g fresh wt^{-1}).

2.6. Comparison of the sensitivity of wheat and D. lanuginosum SODs to temperature

To compare the impact of temperature on SOD stability, leaf extracts prepared from wheat plants propagated at 21/21 °C (day/night) and from leaves of *D. lanuginosum* (KD8 isolate) plants propagated at 35/25 °C were incubated for 1, 3 or 5 h at 25, 35, 45, and 55 °C. Aliquots of each treated extract were centrifuged through a 10 kDa ultra-filtration membrane, and SOD activity was quantified in four replicate samples of each crude extract and in the fraction of each extract that was retained by a 10 kDa ultra-filtration membrane.
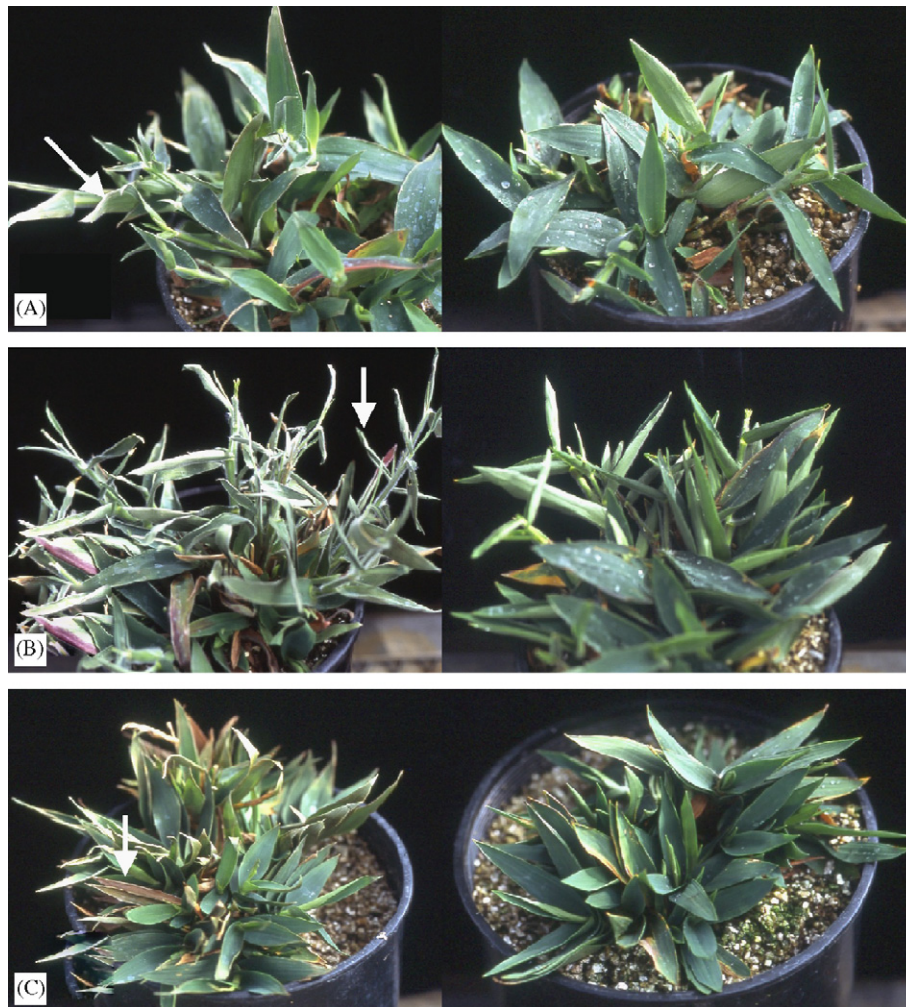

Fig. 1. Phenotypic responses of three *Dichanthelium lanuginosum* biotypes exposed to 58 °C air temperature for 4 h. (A) *D. lanuginosum* collected from a xeric site with active recent geothermal activity at Norris Basin, Yellowstone National Park, Wyoming. (B) *D. lanuginosum* collected adjacent to fumaroles in 40 °C+ soil at Rabbit Creek, Yellowstone National Park, Wyoming. (C) *D. lanuginosum* collected from a non-thermal temperate site at USF.S. Susan Creek Campground, Oregon near the North Umpqua River. Heat shocked plants are on the left of each panel and greenhouse-propagated plants are on the right. Arrow in (B) refers to rolled leaf, an apparent temperature response.

2.7. Impact of temperature on leaf SOD activity and chelating potential

A comparison of the impact of temperature on SOD and chelating activities in wheat and three isolates of *D. lanuginosum* was conducted as follows. Wheat and *D. lanuginosum* plants were propagated in a growth chamber with a 12 h photoperiod and maintained at 21/21 °C (day/night). Control plants were maintained at 21/21 °C throughout the experiment while heat shocked plants were transferred to a growth chamber maintained at 45/35 °C (day/night) at the beginning of the 35 °C dark cycle. Penultimate leaves were removed from plants at specified times for quantification of SOD and chelating activities. Wheat seedlings were treated and harvested at the four-leaf stage. Three independent trials were conducted. The quantities of *D. lanuginosum* leaf tissue only permitted replicate samples at 0 and 24 h.

2.8. Impact of EDTA on SOD activity

Ultra-filtration fractions of leaf extracts from heat shocked and ambient-treated *D. lanuginosum* (KD10) were prepared, and the chelating activity present in the low molecular weight fraction was quantified. Specified quantities of EDTA prepared in water (pH 8.0) were added to aliquots of the SOD-containing fraction retained by the filter, and SOD activity was quantified after incubation for 2 h at 20 °C.

3. Results

3.1. Exposure of plants to elevated temperatures

The three *D. lanuginosum* isolates were exposed to 58 °C for 4 h to determine whether they differed in responses to temperature. Genotypic-dependent responses included no phenotypic response (KD8), leaf curling (KD9), and apparent leaf death in the KD10, the genotype isolated from the non-thermal environment of western Oregon (Fig. 1). In contrast, wheat plants subjected to 45 °C for 8 h showed extensive leaf and plant death (data not shown). Leaf pubescence was observed among the most thermo-tolerant *D. lanuginosum* isolates, KD8 and KD9 (Fig. 2).

3.2. Comparison of wheat and D. lanuginosum SOD activity and stability

To compare the stability of wheat and *D. lanuginosum* SOD activity in crude leaf extracts, SOD activity was quantified after extracts were incubated at selected temperatures for 1–5 h. SOD activity also was quantified in the retained portion of leaf extracts centrifuged through a 10 kDa ultra-filtration membrane because previous research demonstrated that SOD activity was restored after removal of low molecular weight compounds that accumulated after heat shock (Banowetz et al., 2004).

Fig. 2. Enlarged images showing representative differences in leaf surfaces of *Dichanthelium lanuginosum* biotypes in response to exposure to 58 °C for 4 h. (A) *D. lanuginosum* collected from a site with active geothermal activity at Rabbit Creek Yellowstone National Park, Wyoming. Note leaf roll (arrow) and surface pubescence. (B) *D. lanuginosum* collected from a non-thermal temperate site at USF.S. Susan Creek Campground near the North Umpqua River in Oregon. Note the lack of surface pubescence and the death of tissues from the leaf tip and margins (arrows).

Three and 5 h exposure of wheat leaf extracts to 35 °C reduced SOD activity, although 1 h incubation at 35 °C had no significant effect (Fig. 3). Removal of the 10 kDa ultra-filtrates from the crude extracts significantly enhanced apparent SOD activity, although temperature responses remained similar. Exposure of crude wheat leaf extracts to 45 and 55 °C for 1, 3 and 5 h significantly diminished SOD activity, including an approximate 80% reduction following 1 h treatment at 55 °C.

In contrast, SOD activity in crude leaf extracts of *D. lanuginosum* (KD8) remained stable, or increased when the extracts were incubated at 35 and 45 °C (Fig. 4). Exposure of the *D. lanuginosum* KD8 leaf extract to 55 °C for 5 h significantly reduced SOD activity. Removal of the low molecular weight fraction by ultra-filtration of crude extracts significantly increased measurable SOD activity at all temperatures.
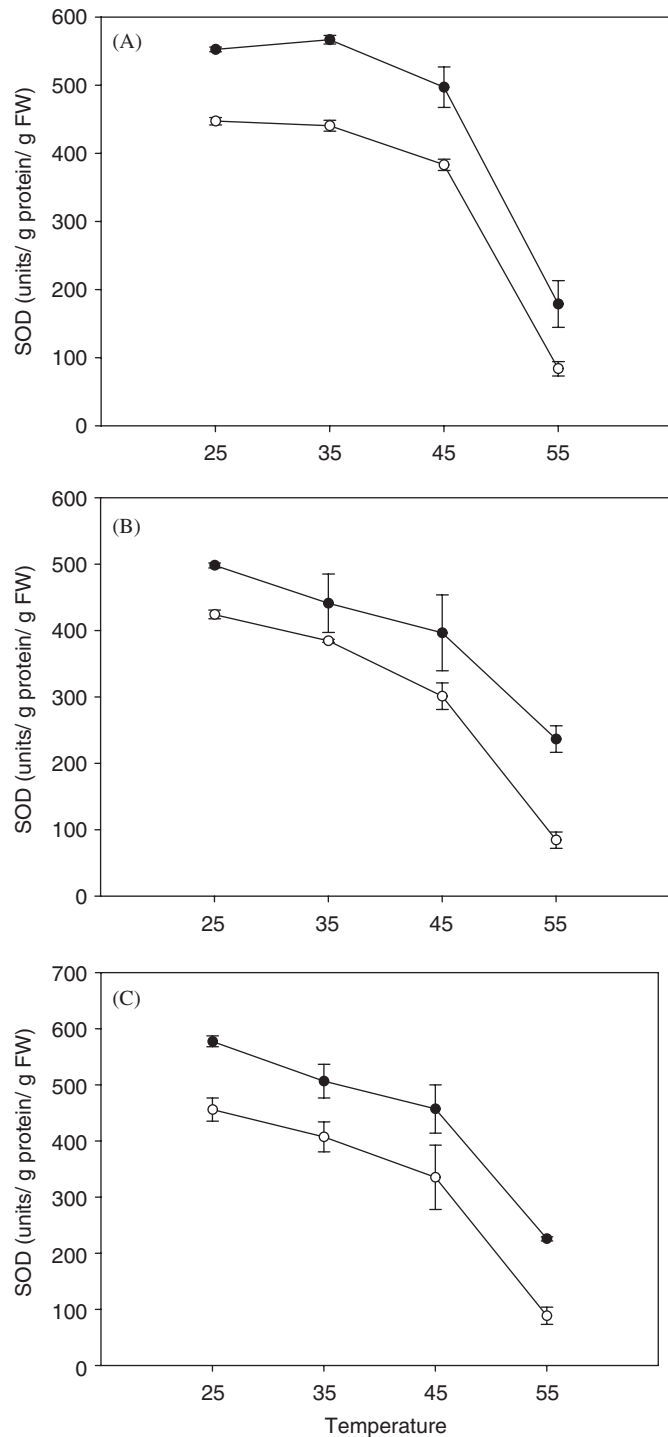
Fig. 3. The impact of ultra-filtration and temperature (°C) on superoxide dismutase (SOD) activity in wheat (*Triticum aestivum*) crude leaf extracts. SOD was quantified in crude leaf extracts (○) and the fraction retained by 10 kDa ultra-filtration (●) after incubation for 1 (A), 3 (B), or 5 h (C) exposure to indicated temperatures.

3.3. Effect of temperature on SOD activity and cellular chelating capacity

Preliminary studies demonstrated that ultrafiltrates contained chelating activity that increased after wheat plants were subjected to elevated temperatures (data not
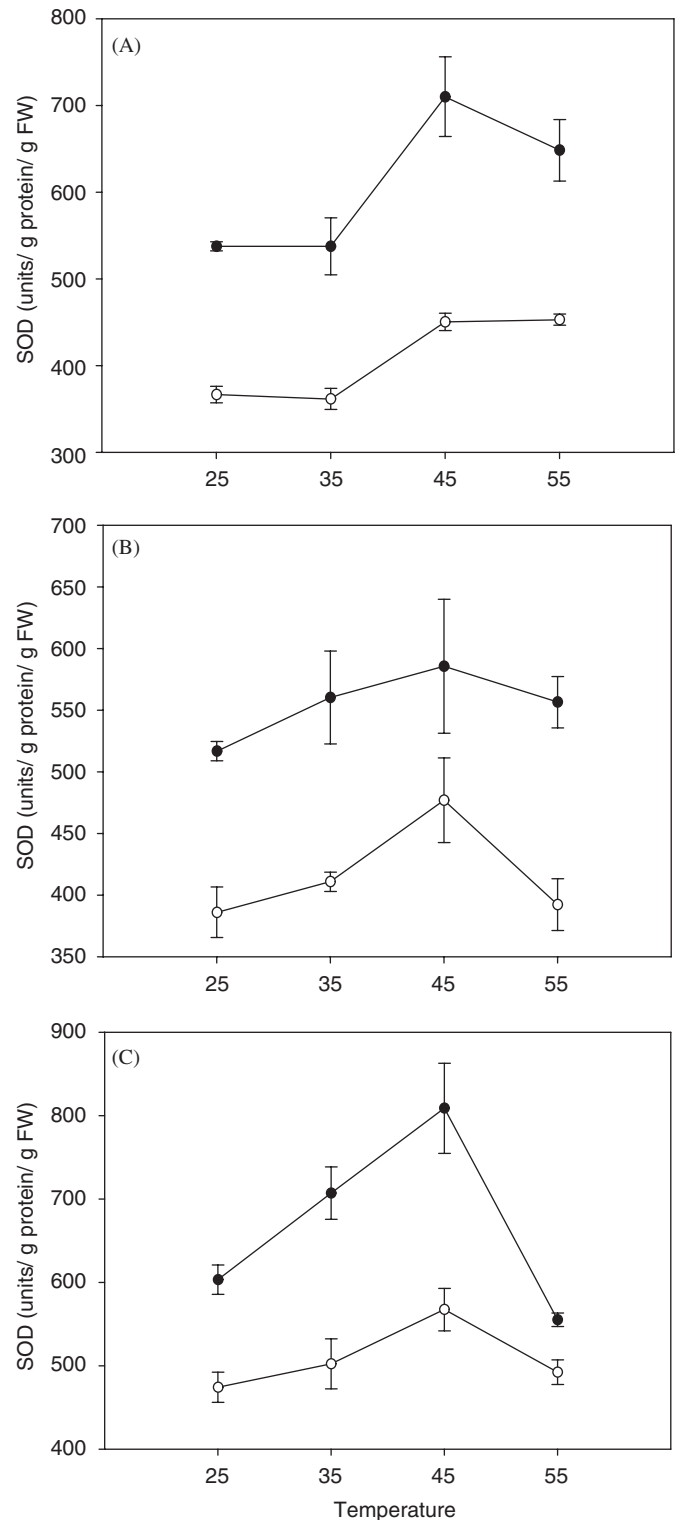

Fig. 4. The impact of ultra-filtration and temperature (°C) on superoxide dismutase (SOD) activity in *Dicanthelium lanuginosum* KD8 crude leaf extracts. SOD was quantified in crude leaf extracts (○) and the fraction retained by 10 kDa ultra-filtration (●) after incubation for 1 (A), 3 (B), or 5 h (C) exposure to indicated temperatures.

shown). To determine whether genotypic differences existed in the accumulation of chelating potential, and whether differences correlated with thermotolerance or

SOD activity, chelating and SOD activities were measured in crude and ultra-filtered extracts from control and heat-shocked plants. Cellular chelating activity increased significantly when the *D. lanuginosum* isolate with reduced thermotolerance (KD10) was exposed to heat shock conditions (Table 1). In contrast, heat shock had no significant impact on chelating activity in the two isolates from thermal environments. Removal of chelating activity by ultra-filtration increased SOD activity in all three isolates. Apparent SOD activity correlated with relative thermotolerance. Leaf SOD activity in KD8 showed no significant change in response to the 45/35 °C heat treatment, but significant decreases in SOD activity were measured in the two remaining *D. lanuginosum* isolates. Leaf SOD activity was consistently lower in KD10, the isolate from a non-thermal environment.

Heat shocked wheat plants showed a significant increase in chelating potential at the 20 h sampling point (Table 2). This time-point corresponded to 8 h exposure at 45 °C because the heat shock treatments were initiated at the start of the 35 °C 12 h dark period. The wheat SOD response to heat shock differed from that of *D. lanuginosum*, showing increased enzyme activity after exposure to the elevated temperatures. Removal of chelating activity by ultra-filtration revealed a significant spike in SOD activity after 20 h into the treatment.

3.4. Impact of metal supplementation and EDTA on SOD activity

To determine whether ultra-filtration of *D. lanuginosum* (KD10) leaf extracts removed critical quantities of divalent cations in complex with chelating substances, SOD activity

Table 1
Impact of temperature on leaf superoxide dismutase (SOD) and chelating potential in selected biotypes of *Dichanthelium lanuginosum*

Biotype	Heat treatment	Leaf SOD activity (units mg protein^{-1} g leaf FW^{-1})	SOD activity in 10 kDa MWCO retentate	Chelating activity in 10 kDa MWCO filtrate (μmol EDTA mg protein^{-1} g leaf fresh wt^{-1})
D. lanuginosum KD8	21/21 °C	154 (52)	272 (27)	4448 (895)
D. lanuginosum KD8	45/35 °C	137 (19)	227 (33)	6156 (957)
D. lanuginosum KD9	21/21 °C	210 (54)	391 (120)	6675 (2358)
D. lanuginosum KD9	45/35 °C	n.d.	230 (104)	7370 (2159)
D. lanuginosum KD10	21/21 °C	n.d.	599 (137)	8699 (421)
D. lanuginosum KD10	45/35 °C	n.d.	95 (77.2)	14,465 (988)

Data are means (standard error), $n = 3$; n.d. = not detected. Greenhouse-propagated plants were transferred to growth chambers maintained at 21/21 °C (day/night) or 45/35 °C [250 μmol m^{-2} s^{-1} PAR with a 12 h photoperiod]. After 24 h, SOD and chelating activities were quantified in each of three penultimate leaves from vegetative tillers. The 10,000 MWCO retentate was the fraction of leaf extract retained by Macrosep® (Pall Filtron Corp.) centrifugal concentrators. The 10,000 MWCO filtrate was the fraction that passed through the ultra-filtration membrane. KD8 isolated from a xeric site in Norris Basin, Yellowstone National Park (YNP); KD9 isolated from a moist site near Rabbit Creek, YNP; KD10 isolated at Susan Creek Campground near North Umpqua River, Oregon.

Table 2
Impact of temperature on wheat (*Triticum aestivum*) leaf superoxide dismutase (SOD) activity, chelating potential, and the removal of chelating activity on SOD activity

Time—fraction	SOD activity 21/21 °C (units mg protein^{-1} g leaf FW^{-1})	SOD activity 35/25 °C	Chelating activity in 10 kDa MWCO filtrate 21/21 °C (μmol EDTA mg protein^{-1} g leaf fresh wt^{-1})	Chelating activity in 10 kDa MWCO filtrate 35/25 °C
0 h—n.f.	52 (41)			
0 h—retentate	125 (15)	—	1190 (174)	—
4 h—n.f.	17 (14)	148 (11)		
4 h—retentate	144 (38)	165 (25)	1269 (19)	1049 (120)
8 h—n.f.	69 (66)	144 (30)		
8 h—retentate	167 (16)	142 (22)	1115 (98)	1201 (22)
12 h—n.f.	117 (20)	259 (56)		
12 h—retentate	136 (29)	299 (81)	1165 (12)	1532 (279)
20 h—n.f.	132 (20)	278 (57)		
20 h—retentate	240 (17)	1772 (1205)	1204 (118)	12,040 (4645)
24 h—n.f.	152 (8)	383 (102)		
24 h—retentate	272 (31)	1063 (304)	1168 (170)	2790 (839)

Data presented as means, $n = 3$, (standard error). Greenhouse-propagated plants were transferred to growth chambers maintained at 21/21 °C (day/night) or 45/35 °C [250 μmol m^{-2} s^{-1} PAR with a 12 h photoperiod]. SOD and chelating activities were quantified in each of three penultimate leaves from vegetative tillers. SOD quantified in the crude extract (n.f.), and the fraction of the extract retained by a 10 kDa ultra-filtration membrane (retentate). Chelating activity quantified in the fraction which passed through ultrafiltration membrane (filtrate) using Cu-chrome azurol (see Section 2).

in the ultra-filtrate retained fraction was quantified after supplementation with Cu^{++}, Zn^{++}, or Fe^{++} at concentrations ranging from 0 to $1000\,\mu M$. Cation supplementation had no effect on SOD activity (data not shown).

When chelating activity was removed from leaf extracts by ultra-filtration and replaced with EDTA, SOD activity in the leaf extract was significantly reduced (Fig. 5). Addition of $500\,\mu M$ EDTA reduced the apparent SOD activity to that measured in the crude extract prior to ultra-filtration. Chelating activity quantified in material that passed through the ultra-filtration material was $1036\,\mu M$ of EDTA equivalents.

4. Discussion

These studies demonstrate genotypic differences in the SOD responses and temperature-associated accumulation of chelating activity exhibited by *D. lanuginosum* collected from geo-thermal sites relative to that of wheat and a *D. lanuginosum* isolated from a non-thermal environment. The role of these responses in enhancing tolerance to oxidative stress encountered in high altitude thermal environments is unknown, although these and previous studies (Perl et al., 1993; Sen Gupta et al., 1993) suggest that reduction in Cu/Zn-SOD activity reduces thermo-tolerance.

The increased chelating activity in response to heat shock was greatest in plants that were most sensitive to heat shock treatments and minimal in *D. lanuginosum* isolates that displayed the greatest thermotolerance. One hypothesis is that the reduced SOD activity observed in some cases resulted from chelation-associated sequestration of Cu^{++}, Zn^{++}, or other metal cofactors required for SOD activity. Zinc is required for structural integrity of Cu/Zn-SOD proteins, while copper plays a catalytic role in the dismutation of superoxide radicals (Beem et al., 1974).
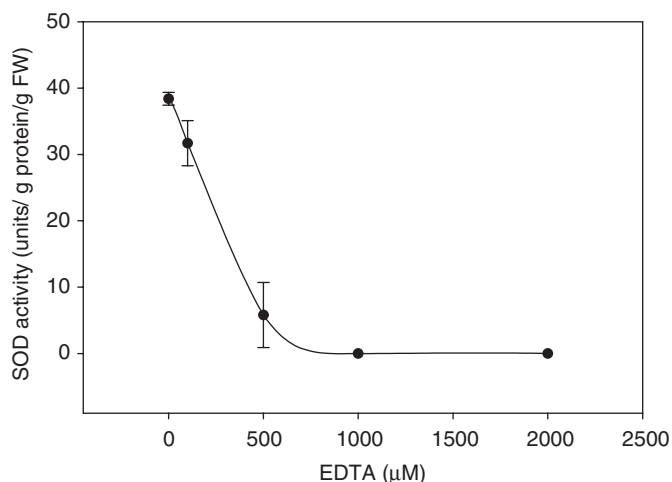

Fig. 5. The effect of ethylenediaminetetraacetic acid (EDTA) on super-oxide dismutase (SOD) activity in 10 kDa ultra-filtration retentates of leaf extracts from *Dichanthelium lanuginosum* (KD10) isolated from a non-thermal environment.

Available levels of Zn impact Cu/Zn-SOD activity in wheat (Hacisalihoglu et al., 2003). Copper availability for SOD activity relies on the activity of copper chaperones because intracellular free copper is undetectable and highly toxic under normal physiological conditions (Rae et al., 1999; Chu et al., 2005). How intracellular copper is sequestered in grasses under normal physiological conditions or whether chelation impacts the availability of copper to the chaperone process is unknown. Removal of chelating activity by ultra-filtration enhanced apparent SOD activity, suggesting a cause–effect relationship between chelating potential and SOD activity.

With the exception of very general molecular weight estimates and the knowledge that chelating activity is not removed by C_{18} adsorption, the nature of the chelating activity remains unknown. Two well-characterized classes of chelating molecules in plants are phytochelatins (PCs) and metallothioneins (MTs; Cobbett and Goldsbrough, 2002). Both are cysteine-containing compounds that sequester metals that cause oxidative damage to plant cell membranes (Hall, 2002), and examples of both have been isolated from grasses (Okumura et al., 1991; Snowden and Gardner, 1993; Yu et al., 1998; Clemens et al., 1999; Charbonnel-Campaa et al., 2000; Ma et al., 2003). Temperature-associated accumulations of PC and MT transcripts have been demonstrated in heat-stressed garlic (*Allium sativum*; Zhang et al., 2005) and rice (*Oryza sativum*; Hsieh et al., 1995). In general, increased PC and MT accumulation are associated with enhanced stress tolerance. In the present study, changes in PC and MT content were not quantified, but accumulation of chelating activity was associated with reduced thermotolerance. In addition, SOD activity declined with increased chelation. In contrast, SOD and PC accumulation increased simultaneously in velvetgrass (*Holcus lunatus*) plants exposed to copper or arsenate (Hartley-Whittaker et al., 2001). One study demonstrated a reduced level of stress tolerance associated with over-expression of a PC synthase gene in *Arabidopsis* (Lee et al., 2003).

Whether SOD has a direct role in genotypic differences in thermotolerance remains to be determined. The observation that SOD levels in KD9 (thermotolerant) and KD10 (non-thermotolerant) *Dichanthelium* isolates were similar at heat shock temperatures suggests no direct correlation. Chelating activity however was significantly greater in the non-thermotolerant genotype. Previous research with *Nicotiana plumbaginifolia* demonstrated that Cu/Zn-SOD mRNA was strongly induced in response to heat shock ($37\,^{\circ}C$) and during recovery from chilling stress (4°), and that these responses were largely independent of light regimes (Tsang et al., 1991). *Nicotiana* Mn-SOD and Fe-SOD transcripts were responsive to chilling stress, but less so to heat shock. Kernodle and Scandalios (2001) showed that Cu/Zn-SOD mRNA from maize, a warm season crop, was induced earlier at $35\,^{\circ}C$ than at $25\,^{\circ}C$ and remained at higher levels. At $40\,^{\circ}C$, the maize Cu/Zn-SOD mRNA levels ranged from negligible to normal.

It is also possible that the accumulation of chelating substances in response to temperature results from the release of previously sequestered chelating substances due to temperature-associated membrane damage. Further isolation and characterization of chelating substances in the ultra-filtrate is needed to identify the source and nature of the chelating activity.

Acknowledgments

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